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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The main objective of this research project has been to apply the T-body approach for the immunotherapy of breast cancer. To this end, we endowed human lymphocytes with anti-erbB2 specificity by transducing them with a tripartite chimeric receptor made of anti-erbB2 scFv-linked through the homeo-domain, trans membrane and endo-domain of CD28 to the cytoplasmic region of the FcRγ chain. In this report we describe an improved protocol for the preparation of a retrovector harboring the chimeric gene and efficient expression of this transgene in human T cells. The ability of the tripartite chimeric receptor to fully activate primary T cells was demonstrated in vitro and in vivo using transgenic mice made with a model receptor. Transgenic mice that express erbB2-specific receptor have been generated and the ability of it's lymphocytes to reject erbB2 expressing tumors will be tested. To avoid tumor escape chimeric receptors of two scFv's from different antibodies were prepared. Using breast cancer xenografts we started to test the anti-tumor potency of erbB2-specific, genetically engineered human lymphocytes in SCID mice. In another pre clinical model, we have expressed the erbB2-specific tripartite chimeric receptor in murine stem cells and are studying their ability to mature into mature effector cells. Altogether, we expect from the model systems described above to establish the optimal conditions of using T-bodies for breast cancer therapy.

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Abbreviations:

BM – Bone marrow

CR – Chimeric receptor

DTH – Delayed type hypersensitivity

FcRγ - Gamma chain of the Fc receptor

FγG – Fowl gamma globulin

GFP – Green fluorescent protein

GMP - Good manufacturing practice

IL-2 – Interleukin 2

IL-2R – IL-2 receptor

^{125}I-UdR- Radioactive Iodo deoxy uridine

LAK – Lymphokine activated killers

mAb – Monoclonal antibody

MHC – Major histocompatibility complex

NK – Natural killer cells

PBL – Peripheral blood lymphocytes

RN – RetroNectin^{TM}

ScFv – Single chain Fv of an antibody

SCID – Severe combined immune deficiency

T-body – Chimeric receptor expressing T cells

TCR – T cell receptor

Tg - Transgenic

Tg8.7 – A particular transgenic mouse expressing TNP-specific CR

TIL – Tumor infiltration Lymphocytes

TNP - Trinitrophenol

VH – The variable domain of the antibody heavy chain

VL - The variable domain of the antibody light chain

WT – Wild type

INTRODUCTION

In this proposal we suggest a novel approach for the immunotherapy of breast carcinoma. The difficulty of treating metastatic breast cancer with conventional therapy, combined with the presence of defined tumor - associated antigens on breast tumors, makes this malignancy an ideal candidate for an immunotherapeutic approach to treatment.

One method developed for the immunotherapy of cancer is to remove lymphocytes from tumors obtained at surgery or biopsy, expand them *ex vivo* in the presence of lymphokines, and reinfuse these cells into the patient. Treatment of cancer by the infusion of such autologous tumor infiltrating lymphocytes (TIL) has produced clinical responses in some patients. Although several explanations can be proposed for the limited response thus far, one possibility is the lack of specificity of the reintroduced lymphocytes. In addition, this technique is limited by the difficulty in obtaining specific TIL for many histologic types of cancer (including breast cancer). In contrast, many monoclonal antibodies that bind tumor-associated antigens shared by tumors of similar histology (e.g. Anti-HER2 (anti-erbB2) antibodies) have been described. These monoclonal antibodies can be attached to either a cytotoxin or to an antibody or growth factor to redirect cytotoxic T cells. However, most clinical attempts using such immunotoxins have not fulfilled expectations. Their therapeutic efficacy is restricted to blood borne tumors, primarily because solid tumors are not sufficiently accessible to antibodies. The recently used antibodies for breast cancer therapy, anti-HER2 antibodies (Herceptin), show benefit to patients yet are not curative and 1n order to optimize their anti-cancer effect the addition of inhibitors of cell division (e.g. Taxol) is recommended.

Cancer patients usually mount a poor -if any- immune response against their own tumors due to poor tumor immunogenicity, escape from immune-attack and an immunosuppressed state common to many cancer patients. Several approaches have been attempted to enable the immune eradication of tumor cells. These include various methodologies to augment, non-specifically or specifically, the host immune response and treatment with specific anti-tumor antibodies. Non-specific treatments, such as the use of LAK (lymphokine-activated killer) cells, are not effective in all types of cancer. The requirement for the co-injection of large amounts of IL-2 causes severe side effects that often require the cessation of treatment. The use of antibodies in passive immunotherapy is often of limited efficacy, not only because of the difficulty in identifying true tumor-specific antigens, but also because of poor tumor penetration, and the short half-life of the antibodies.

Our group has pioneered the "T body" approach, a novel approach for cancer therapy. This approach has several advantages over traditional immunotherapeutic methods. We have joined the two approaches of adoptive immunotherapy and immunotoxin therapy to genetically engineer an improved 'immunocytolysin', which is an antibody recognition unit in the form of single chain variable (scFv) region attached to a cytotoxic T cell signaling molecule. Genes for chimeric T cell receptors have been constructed containing the coding sequence of an antibody-derived scFv directed against a tumor associated antigen, attached to the transmembrane and intracytoplasmic sequences of a T cell signaling molecule. These genes are then transfected into cytotoxic T cells, thereby conferring upon them the ability to specifically recognize and kill tumor cells. The chimeric scFv receptor (scFvR) design we have developed combines antibody recognition and T cell signaling in one continuous protein and has been used to endow murine and human cytotoxic cells with non-MHC-restricted, antibody-derived specificity. This T-body approach combines the advantage of antibody specificity with the homing, tissue penetration, for the immunotherapy of breast cancer. Accordingly, the research work will focus on the preparation and efficient expression of breast-cancer specific chimeric receptors and the establishment of in-vivo pre-clinical model in which the different aspects of the T-body approach could be evaluated and optimized.

Body

In the previous, first annual report, we described the construction of tripartite chimeric receptor (CR) genes made of scFv of an antibody as the extracellular recognition unit, the homeo-domain and trans -membrane of CD28, and the intracellular region of CD28 linked to the intracellular part of the FcRγ as the cytoplasmic co-signaling and signaling regions (1). Such tripartite CR expressed in murine cells (hybridomas or transgenic mice) could redirect the T cells with antibody specificity and trigger them for full activation. We also reported on the development of chimeric receptors specific to the erbB2 (HER2/Neu), the growth receptor over-expressed on more than 30-40% of human breast carcinoma. In parallel, we developed an efficient transduction procedure to express the tripartite CR in human PBL.

In the present, second annual report, we further describe the characterization of the transgenic mice harboring a model TNP-specific tripartite receptor. We report for the first time on our successful efforts to establish transgenic mice harboring erbB2-specific CR. We present in detail our improved protocol for the transduction of human lymphocytes with the erbB2-specific CR using the pBullet retrovector pseudotyped with the gibbon ape leukemia virus envelope (2). We describe our initial attempts to evaluate, in SCID mice, the in-vivo potential of the T-body approach, using human breast xenograft and genetically modified erbB2-specific human PBL. Finally, we report on the establishment of a system to transduce murine bone marrow derived stem cells with the erbB2-specific CR.

Full activation of primary murine lymphocytes in mice transgenic to TNP-specific chimeric receptor

The transgenic (Tg8.7) mice, expressing high levels of the TNP specific tripartite CR, were described in the previous report. These mice serve as an in-vivo model to evaluate the potency of the tripartite receptor, the receptor of choice that is being used in all our studies aimed to establish the potential of the T-body approach for breast cancer therapy. Here we describe additional data collected during the second year.

Stimulation of IL-2 production
IL-2 as well as other cytokines production is an important component of the T-body's anti-tumor activity. Upon interaction of the T-bodies with the tumor target we expect such cytokines not only to provide growth factors that sustain the T cell reactivity, but also to attract other cells and induce a local inflammatory response, which eventually should cause the elimination of the tumor. We have expected the chimeric receptor to signal the T cell activation in non-MHC restricted manner, and indeed, as demonstrated in the experiments detailed below, the mere binding of the tripartite CR to its plastic immobilized TNP hapten is sufficient to fully activate the T cells.

Figure 1 shows that stimulation of purified (90%) naïve resting splenic T cells of Tg8.7 mice on plastic-bound TNP-FγG for 24 hr induces high levels of specific IL-2 production in response to tripartite CR signaling.

Stimulation of proliferation by anti-TNP tripartite CR.
Naïve T cells stimulated only through the TCR enter into a state of anergy which is manifested by their inability to proliferate in response to repeated stimulation. Thus, proliferation is the most indicative parameter for CD28 mediated co-stimulation. Proliferation assays of naïve resting splenic T cells of Tg8.7 mice stimulated at $2x10^5$ cells/ml in microtiter plates for 48 hr (**Fig. 2**) indicates that signaling via tripartite CR specifically induces a significant degree of proliferation. These levels of proliferation were markedly higher than those induced by stimulation with anti-CD3 antibodies at effective concentration, as determined in control, wild-type mice (WT). This type of experiments clearly establishes the co-stimulatory signaling mediated by the tripartite CR.

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Rescue from apoptosis in cells activated via anti-TNP tripartite CR
Rescue of apoptosis caused by the lack of proper T-cell signaling or due to antigen-induced cell death, is mediated by the co-stimulatory signaling through CD28 (3-5). Analysis of apoptosis in bulk splenocytes and in purified (90%) splenic T cells from Tg8.7 mice stimulated at 10^6 cells/ml in 24-well plates for 72 hrs indicated that stimulation via tripartite CR is effective, as expected from the CD28 co-stimulatory domain (**Fig. 3 and Fig. 4**).

Up-regulation of the anti-apoptotic transcription factor bcl-X$_L$ by stimulation via anti-TNP tripartite CR
The rescue from antigen-induced apoptosis by CD28 is mediated through the bcl signaling pathway. The bcl-X$_L$ gene has been reported to enhance the intrinsic ability of lymphocytes to resist apoptosis (3). Indeed **Figure 5** shows increased bcl-X$_L$ expression in primary T cells, purified from the Tg8.7 mice and stimulated via anti-TNP tripartite CR.

Up-regulation expression of IL-2Rα chain (CD25) by stimulation via anti-TNP tripartite CR
One of the most common markers for T cell activation is the up-regulation of the IL-2Rα chain (CD25) of the IL-2 receptor. Stimulation of purified (90%) splenic T cells from Tg8.7 mice for 60 hr with anti-CD3 mAb generated a low degree of IL-2Rα T cells, whereas a larger number of IL-2Rα T cells were induced by stimulation via anti-TNP tripartite CR (**Fig. 6**).

Summary of in-vitro experiments
Altogether, these in vitro results demonstrate that stimulation of T cells via the tripartite CR provides both FcRγ - mediated (signal I) and CD28 - mediated (signal II) triggering (6).

In-vivo, antigen-specific responses in CR transgenic mice
The TNP hapten is capable of eliciting delayed type hypersensitivity (DTH) response when applied by skin sensitization. We have therefore compared the ability of the Tg8.7 (generated with TNP-specific CR) to mount TNP-specific DTH without pre-sensitization.
Groups of mice were pre-treated with cyclophosphamide and two days later sensitized with the antigen in their shaved backs (skin painting) Mice were challenged five days later by painting the right pinnae with the antigen and the left one with the solvent only. One hour later mice were pre-treated with 5-fluorodeoxyuridine and an intravenous pulse of 2 μCi ^{125}I-UdR was given into the tail vein. 24 hr after ^{125}I-UdR administration, the mice were killed and the pinnae cut off at the hairline (7). The ear reaction reflects a T-cell-dependent DTH response.
The preliminary study depicted in **Figure 7** shows that there was no difference between WT and Tg mice in their ability to elicit DTH response. More importantly - a weak response of the transgenic mice has been observed upon challenging the mice only with the antigen without pre-sensitization. The ratio of radioactivity intake between the ears in the Tg mice is higher than in the WT mice and reflects the extent of inflammatory reaction elicited by the antigen challenge (**Fig.7**). These responses were associated with an extensive mononuclear and lymphoid cell infiltration (**Fig. 8**).
We thus conclude that the primary T cells harboring the tripartite chimeric receptors are capable of responding in-vivo. These results indicate the feasibility of using the Tg mice and their cells in experimental models aimed to establish the ability of T bodies to reject established breast tumors.

Generation of Transgenic Mice made of anti-erbB2 tripartite CR

For these experiments, the N29-scFv-CD28-gamma chimeric receptor gene was used where N29 is one of the anti-erbB2 monoclonal antibodies (mAb) we have been using throughout this research project.

Transgenic mice were generated by pronuclear microinjection of (BALB/c x C57BL/6)F2 embryos with a construct for expression of (CD2)-N29-CD28-γ. The CD2 tissue-specific promoter (8) allows the gene under its control to be expressed exclusively in T cells and NK cells. The vector we were using proved itself efficient in the Sp6 (anti-TNP) based CR we described above and in the previous report. DNA for microinjection was prepared as follows: linearized scFvR transgenes were separated from the rest of the vector sequences by restriction enzyme digestion and gel electrophoresis in low-melting point Agarose. The transgene containing DNA fragments were prepared by melting the Agarose slice in a 10-fold volume of low-salt buffer. Finally, the transgene DNA were purified by affinity chromatography using Elutip-d columns.

Identification of Transgenic Founders. Genomic DNA was isolated from tail tissue of founder mice and was analyzed for integration of transgene by PCR amplification of transgene specific sequence with co-amplification of an endogenous CD3δ specific sequence as an internal control.

Out of 70 founder mice obtained, six transgenic founder mice (3 females and 3 males), have tested positive for integration of N29 (anti-ErbB2)-CD28-γ transgene (**Fig. 9**). Reconstruction experiments indicate that between one and twelve copies of transgene are integrated in the germ line of transgenic founder mice.

Surface Expression of the erbB2-Specific Chimeric Receptor in the Transgenic Mice. Surface expression of the anti-erbB2 chimeric receptor in the six mice harboring the tripartite chimeric gene was evaluated by testing their PBL for staining using polyclonal rabbit anti-N29 antibodies. The female mice with the highest copy number also scored positively (**Fig. 10**). Another mouse showed weak staining. These two mice are being bred now and their offspring will be tested for germ line transmission and expression.

Construction of a tripartite chimeric receptor made of a new anti-erbB2 mAb

So far, most of our studies used a chimeric receptor made of scFv of the N29 anti-erbB2 mAb as their recognition unit (9). This antibody developed in the laboratory of Prof. Y. Yarden, was found to be effective in slowing the growth of human tumors in nude mice (10). It does not react with recombinant erbB2 made in bacteria and is considered to recognize carbohydrate, or carbohydrate-containing epitope in the extracellular part of erbB2 molecule (Y. Yarden; personal communication). Human T-bodies, expressing the tripartite CR made of the N29 scFv, injected intra-tumorally into prostate carcinoma xenograft growing in SCID mice was capable of slowin the growth of this tumor and even of curing significant number of these mice. Nevertheless, in a few mice, tumor growth was resumed after a certain period. When removed and analyzed for surface erbB2 expression, it was interesting to find out that the recovered tumor cells were not stained by the N29 mAb, but stained quite well by L96, (**Fig. 11**) another anti-erbB2 mAb produced in Yarden's laboratory (11). Based on such observations, we believe that the erbB2-specific T bodies provide a growth advantage to tumor cells that do not express the N29 epitope. Whether this immunoselection is due to pre-existing variants that do not express the N29 epitope or due to cells that ceased to express it as a result of selective pressure is being currently studied. Nevertheless, we have concluded from this behavior that for clinical trials it may be beneficial to use another anti-erbB2 mAb in the CR context. Consequently we elected the L96 mAb that strongly stained surface erbB2 on several human adenocarcinomas (see **Figure 12** for comparison of the staining of the human breast adenocarcinoma SKBr3 by N29 and L96). Accordingly, we have cloned the VH and VL from the L96 hybridoma, prepared scFv and used it in the framework of the tripartite CR. The CR gene was inserted into the pBullet retrovector and we are currently preparing a packaging cell producing a high virus titer for the infection of T cells.

Optimization of the transduction procedure and chimeric gene expression in human lymphocytes

In the previous report we described the procedure that we developed for the preparation and selection of packaging cells producing high titers of the pBullet vector harboring the chimeric receptor and GFP genes. We have now improved and optimized the transduction procedure so as to reproducibly obtain efficient (40-80%) and stable gene transfer into human PBL. The protocol below describes this procedure which may serve as the prototype to be up-graded to GMP conditions:

Protocol for transduction of human lymphocytes
Packaging Cell Lines
Cell lines used are the ecotropic GPE86 (ATCC), amphotropic PA317 (ATCC), the PG13 expressing the gibbon ape leukemia virus envelope (GALV env) (12). The 293T cells were obtained from R. A. Willemsen (Daniel den Hoed Cancer Center, Rotterdam, The Netherlands). All cells were cultured in DMEM medium (GIBCO-BRL) supplemented with 10%FCS (GIBCO-BRL), L-glutamine solution (2mM), Sodium Pyruvate 1mM, 100 units/ml Penicillin and 100μ/ml Streptomycin (Biological Industries Israel).

Antibodies and Reagents
Anti-human CD3 (OKT3) antibody was purified from hybridoma cell culture supernatant.
Anti-CD28.2 was purchased from PharMingen. SP6 is an anti–TNP monoclonal antibody (mAb) and 20.5 an anti-Sp6 idiotype mAb were provided by Dr G. Kohler (13). Fluorescein labeled anti-mouse Ig antibodies were purchased from Sigma. IL-2- Human recombinant IL-2 (Chiron, Amsterdam, The Netherlands). RetroNectine (RN) (Takara Shuzo Ltd. Otsu, Japan).

Preparation of packaging cells
$3x10^5$ GPE86 cells and $2x10^5$ PA317 cells were plated together in a 100mm plate (NUNC). A day later, medium is changed and transfection is performed by adding 20 μg of DNA in $CaPO4$ (Mammalian Transfection Kit, Stratagene). After 24 hours the plate is washed with PBS and supplemented with fresh medium. 48-72h later, when the culture is nearly confluent, viral supernatant can be collected.
Pseudotyping was accomplished by infection of PG13 cells. $2x10^5$PG13 cells are plated in a 100mm plate; the next day the medium is replaced with 5ml of viral supernatants in the presence of 4mg/ml of Polybrene at 37^0C, 7.5% CO_2 for 7 h. 48h later the infection efficiency is evaluated. All vector-containing retroviral supernatants described in this study were harvested after a 24h incubation of near-confluent cells grown in 5ml fresh medium in a humidified incubator at 32^0C, 7.5% CO_2.

Transduction of human lymphocytes
Activation of Lymphocytes
Fresh peripheral blood lymphocytes (PBL) of healthy donors were isolated by centrifugation through Ficoll Paque Plus (Pharmacia Biotech), and cultured in RPMI 1640 medium (GIBO-BRL) supplemented with 10%FCS, L Glutamine solution 2mM, 100 unit/ml Penicillin 100mg/ml Streptomycin and 50μM 2-mercaptoethanol. Cells, (4ml of10^6/ml /well in a 6-well plate) were stimulated for 3 days, on Falcon non-tissue culture treated 6-well plates, pre-coated with anti-CD3+anti-CD28 antibodies. Immobilization of the antibodies was performed by adding 2.5 ml/well of antibody (1.2μg/ml of each in PBS) to the cells for overnight at 4^0C, washed with PBS and blocked with 1% BSA in PBS for 20 min at 37^0C.

Lymphocyte transduction
1.5ml/well of viral supernatant supplemented with 100 U/ml of IL-2 is added of RN coated 6 well plate and incubated for 30 min.at 37^0c. Activated lymphocytes were harvested from the stimulating plate, washed and

resuspended, 2.5×10^6 cells in 1.5ml of viral supernatant added to the RN plate. After 4-6h at 37^0C 7.5% CO_2 the viral supernatant was gently removed and replaced with 4ml of RPMI-FCS+ 100U/ml IL-2 medium and incubated overnight at 37^0C 5% CO_2. The same transduction process was repeated on the next day. On the following day lymphocytes were harvested by vigorous pipeting and washing of the wells to obtain all cells. The cells were resuspended in RPMI-FCS medium with 350U/ml of IL-2, and incubated in 37^0C 5% CO_2. RN coated plates were prepared by incubating non tissue culture treated 6-well plate (Falcon) with 4ml/well of RN 12μg/ml over night at 4^0C, washed with PBS and blocked with 1% BSA in PBS for 20 min at 37^0C.

Analysis for transgene receptor expression

Flow Cytometry

Cells were analyzed using a FACScan flow cytometer (Becton Dickinson). Green fluorescent protein (GFP) was measured on FL1 530nm emission filter and 488nm excitation. Viability was determined by assessing Propidium Iodide (PI) (Sigma) staining on FL2 585nm emission filter and 488nm excitation. Expression of scFv (Sp6) on the surface of the cells was evaluated by immunofluorescence staining using 20.5 anti-Sp6 mAb and R-Phycoerythrin (PE) labeled anti-mouse Fab' antibody, measured on FL2.

Cells were sorted on FACSsort Plus (Becton Dickinson) according to GFP fluorescence. Cloning to a single cell/well was accomplished by sorting of positive cells into 96 well plates.

Cell Proliferation

96 well plates (non tissue culture treated, NUNC) were coated for overnight with 2mg/well of antigen (TNP-FγG), or irradiated (12,000 rad) target cells washed with PBS and blocked with 1% BSA or medium+FCS on the following day.

The transduced lymphocytes were washed twice and re-suspended in culture medium RPMI- FCS with IL-2. 10^5/ml transduced lymphocytes were plated on antigen-coated plate or with target cells. At different time points, 50 μl of the cell culture were taken out to be tested for cell proliferation by MTT assay (14).

Generation of murine cancer target expressing the human erbB2 antigen

In order to determine the ability of murine cells harboring the tripartite, erbB2-specific chimeric receptor genes to reject erbB2 expressing tumor cells, we have transfected the B16, C57BL/6 metastatic melanoma with the human erbB2 gene in pcDNA vector. Following selection in G-418 and a few cycles of sorting, using anti-erbB2 mAb, a clone, over-expressing surface erbB2 was obtained (**Fig.13**). This clone maintained its tumorogenic features in the C57BL/6 mice and manifested somewhat faster growth both in mice and tissue culture.

Establishment of human breast tumor experimental model in SCID mice

In addition to the establishment of breast cancer xenografts from patients' specimens, which is an on-going undertaking in the lab. (done in collaboration with Sheba Medical Center), we have been using the well established SKBr3 breast carcinoma cell line as a xenograft in female SCID mice. The SKBr3 highly expresses the erbB2 target antigen as judged by immunofluorescent staining with both N29 and L96 mAbs (see **Fig. 12**). When transplanted into the fat pad of the mammary gland, together with Matrigel, the tissue cultured SKBr3 produced tumors (**Fig. 14**) in estrogen independent manner.

Additional human breast cell lines (BT-474 and MCF-7) are being adopted to in-vivo growth in SCID mice. These cells are estrogen-dependent. MCF-7 is especially interesting because the level of surface expression of erbB2 is medium-to-low in comparison to the other xenografts and it is of interest to evaluate the in-vivo effect of the T-bodies in relation to the level of erbB2 expression on its target cells.

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Effect of the erbB2-specific T-bodies on breast tumor xenografts in vivo (preliminary experiments)

In a preliminary experiment, erbB2-specific human T lymphocytes were injected into the SKBr3 xenograft growing in the mammary of SCID mice in the absence and presence of a continued supply of IL-2. After 10 days, the tumors were removed, fixed and analyzed histologically. As can be seen in **Figure 15**, the human xenograft grew in the fat pad or in some occasions in the mammary gland as an adenocarcinoma. An inflammatory response could clearly be noticed, and in certain areas tumor destruction is evident. These results are encouraging and further, more extensive, studies are currently beingcarried out in accordance with the approved Statement of Work.

Expression of chimeric receptor genes in murine bone marrow derived stem cells

Another way to obtain mature primary T cells harboring the chimeric receptor genes is by transducing murine bone marrow stem cells with the chimeric receptor gene ex-vivo, implant these cells into irradiated mice and let the stem cells differentiate into mature cells in-vivo. In this approach, feasible in cases that require total body irradiation and regeneration of the hematopoietic system by stem cell engraftment (such as in the case of advanced, spread breast cancer), the tripartite receptor is especially an attractive component. One can foresee a situation in which part of the reconstituting stem cell graft will include CR expressing cells which upon maturation in the host will home to and reject residual cancer cells.

For transduction of the murine bone-marrow derived stem cells we have used erbB2 and TNP specific chimeric genes in the pBullet retrovector as we have used for lymphocytes, but, rather than using the CMV promoter, we used the PGK one that operates better in murine cells. Packaging cells producing high titer viral supernatants were prepared.

For transduction, mice were treated with 5-Fluorouracil and 48 hrs later, bone marrow (BM) cells (Sca$^+$, Lin$^-$) were prepared from the murine long bones and separated on Ficoll. Cells were stimulated in culture in the presence of IL-3, IL-6 and SCF for 48 hrs and then co-cultured on irradiated packaging cells in the presence of the cytokines and polybrene for another 48 hrs. Alternatively, the stimulated BM stem cells were centrifuged with the retrovector supernatants in the presence of protamine at 2000 rpm for 2hrs and then cultured again with the cytokines. The degree of transduction was evaluated by GFP fluorescence and was found that up to 80% in the co-culture procedure and up to 30% by the centrifugation procedure.

The transduced cells were analyzed for colony formation in methylcellulose selective medium to assess their hematopoietic potential, cellular differentiation and GFP expression. Most of the transduced cells were injected i.v. into lethally irradiated (900 rad) mice, and engraftment has been evaluated at monthly intervals by looking for surface staining of peripheral blood cells using antibodies against the different hematopoietic lineages.

By the time this report was prepared, we could observe gene transfer into colonies of megakaryocytes, reticulocytes, as well as myeloid and lymphoid lineages. In the mouse sera we could get 20-50% of the peripheral blood cells expressing GFP by 8 weeks following the stem cell transfer. In some cases, the GFP maker was found at 9 months following engraftment. The level of engraftment of T cells and their expression of the chimeric receptor is being studied.

Progress of research in relation to the Statement of Work:

Task 1+2 (Months 1-12): *Cloning of anti-erbB scFv and constructing of chimeric receptors*
        All work has been completed (described in the first annual report)

Task 3 (Months 3-18): *Functional expression of chimeric receptors in lymphocytes and cell lines*
        Most of the work has been completed. Following our success in expressing the CR genes in human lymphocytes, we do not consider it necessary to go back to the rat mast cell line (RBL).

Task 4 (Months 1-24): *Establishing in vivo model systems*
Accomplished: 1. Screening of breast cancer lines for tumor antigens
               2. Preparation of transgenic mice

On-going: 1. Growth of breast cancer xenografts in SCID mice
               2. Human model in SCID mice
               3. Transduction of bone-marrow stem cells and engraftment. In this sub-task we have diverted from our intention to work on human stem cells because they do not mature into effector cells in the SCID mouse model. As described above, we study murine stem cells.

Task 5 (Months 12-36): *Therapeutic model in mice. Analysis and optimization*
Accomplished: 1. A continued supply of IL-2 is required to maximize the therapeutic potential of T-bodies

On-going: 1. Long term effect of T-bodies
               2. Systemic application to advanced tumors

To be initiated: 1. Analysis of sub-populations
               2. In-vivo fate of T-bodies and development into memory cells

KEY RESEARCH ACCOMPLISHMENTS

- Construction of a tripartite T cell receptor made of scFv-anti-ErbB2-CD28-FcεRγ

- Construction of a tripartite T cell receptor using heregulin as the targeting agent

- Construction of transgenic mice expressing the gene for a tripartite scFv-anti-erbB2-CD28-FcεRγ

- Full functional expression of the tripartite chimeric receptor in transgenic mice

- Production of a retrovirus that can efficiently transduce anti-ErbB2 targeting agent tripartite receptors.

- Functional expression in human lymphocytes of erbB2-specific chimeric receptor

- Establishment of pre-clinical model using human breast cancer xenografts in SCID mice (on-going)

- Expression of the tripartite, anti-erbB2 chimeric receptor in murine stem-cells

REPORTABLE OUTCOMES

<u>Review article</u>: Eshhar, Z., Waks, T., Bendavid, A., and Schindler, D. G. Functional expression of chimeric receptor genes in human T cells. J. Immunol. Methods, 248: 67-76, 2001

<u>Abstract</u> presented at the 11th International Congress of Immunology, Stockholm, July, 2001:
Eshhar, Z., Bendavid, A., Waks, T., Morvinski, D., and Schindler, D. G., Full activation of naive T cells via a transgenic tripartite chimeric receptor.

Partial fulfillment of Ph.D. thesis for Dr . Feigelson

Partial fulfillment of Ph.D. requirement of Alain Bendavid

Development of transgenic mouse strains with tripartite chimeric receptors specific to: I. TNP; II. ErbB2.

Acquired a Grant from the EC 5th Framework program in Quality of Life, The Cell Factory Key Action

CONCLUSIONS:

In this reporting period we have accomplished the construction of tripartite receptors consisting of a targeting molecule such as a scFv or a ligand, a portion of the co-stimulatory molecule CD28, and the signaling portion of the cytoplasmic domain of the Fcε receptor γ chain. We have optimized the expression of these genes as functional surface receptors in the splenocytes of transgenic mice, mouse stem cells, and human lymphocytes. We have established a model for human breast cancer xenograft in SCID mice and started to evaluate the function and therapeutic potential of human lymphocytes bearing the erbB2-specific chimeric receptor. It is towards this aim that most of our research efforts will be directed in the coming year.

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APPENDIX

1. **Experimental Data**
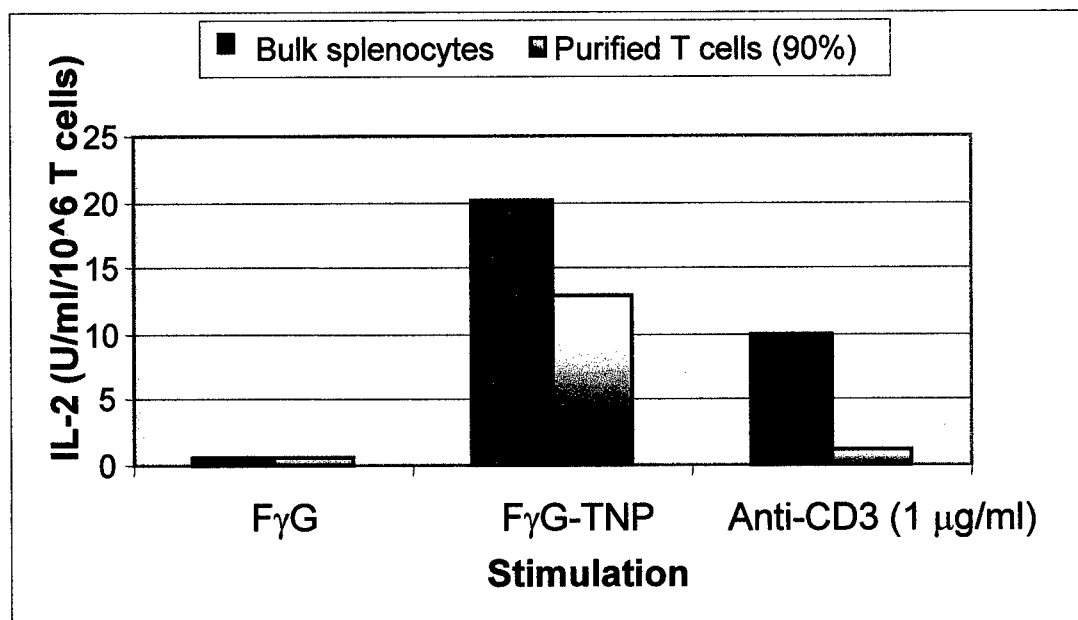2. **Publication**

Fig 1. *Stimulation via SP6-CD28-γ induces APC-independent IL-2 production in purified (90%) unprimed naïve splenic T cells of Tg8.7 mice (24 hr).* Bulk and purified splenic T cells were cultured in plates immobilzed with FγG (10 μg/ml), FγG-TNP (10 μg/ml) and anti-CD3 (1 μg/ml). SN were harvested and tested for IL-2 activity.
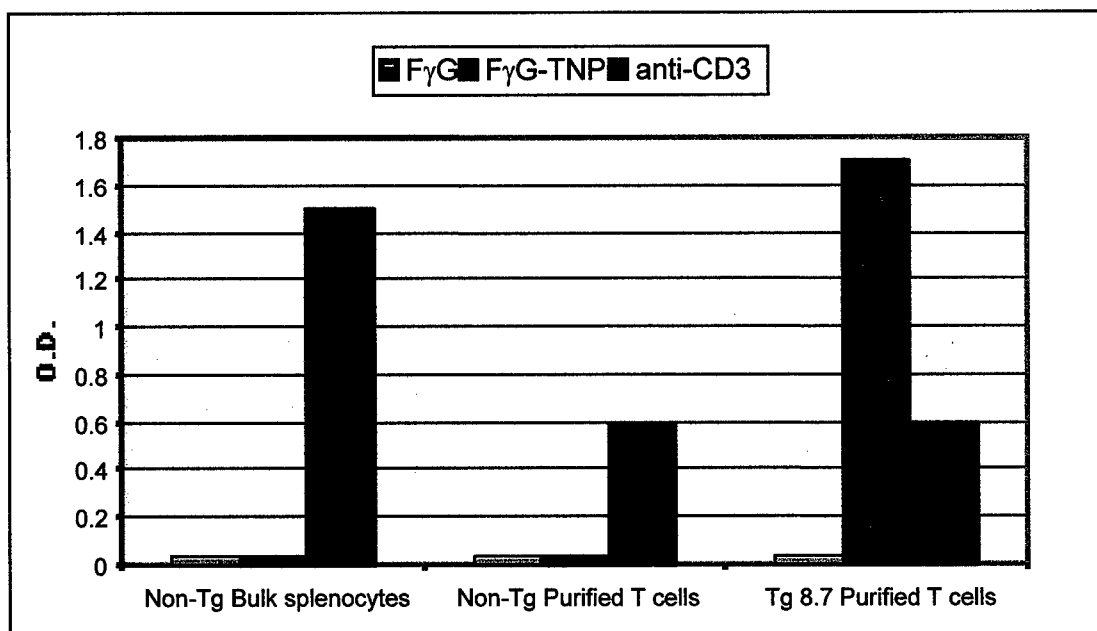

Fig 2. *Unprimed naïve T cells (90%) from Tg8.7 mice proliferate in response to stimulation via Sp6-CD28-γ.* Bulk and purified splenic T cells (2×10^5 cells/ml) were cultured in 96-well flat-bottom microplates immobilzed with FγG (10 μg/ml), FγG-TNP (10 μg/ml) and anti-CD3 (2 μg/ml). After 48 hrs, proliferation was assessed via XTT assay.
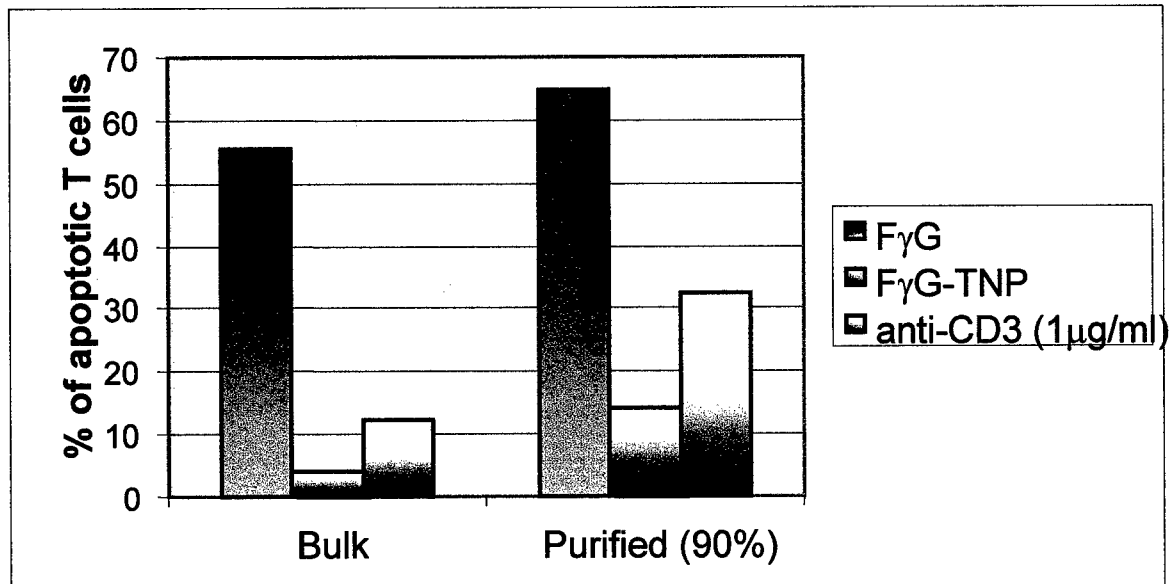
Fig 3. *Stimulation via SP6-CD28-γ induces rescue from apoptosis in purified (90%) unprimed naïve splenic T cells of Tg8.7 mice (72 hr).* Bulk and purified T cells were cultured in 24-well plates that had been coated with the indicated reagents. After 72hrs, cells were stained with FITC-Thy1.2 and fixed. PI and Rnase were added for cell cycle analysis. Percent of apoptosis was measured by FACS analysis on Thy1.2+ gated cells and calculating the percentage of cells displaying sub-G_1/G_0 DNA content.
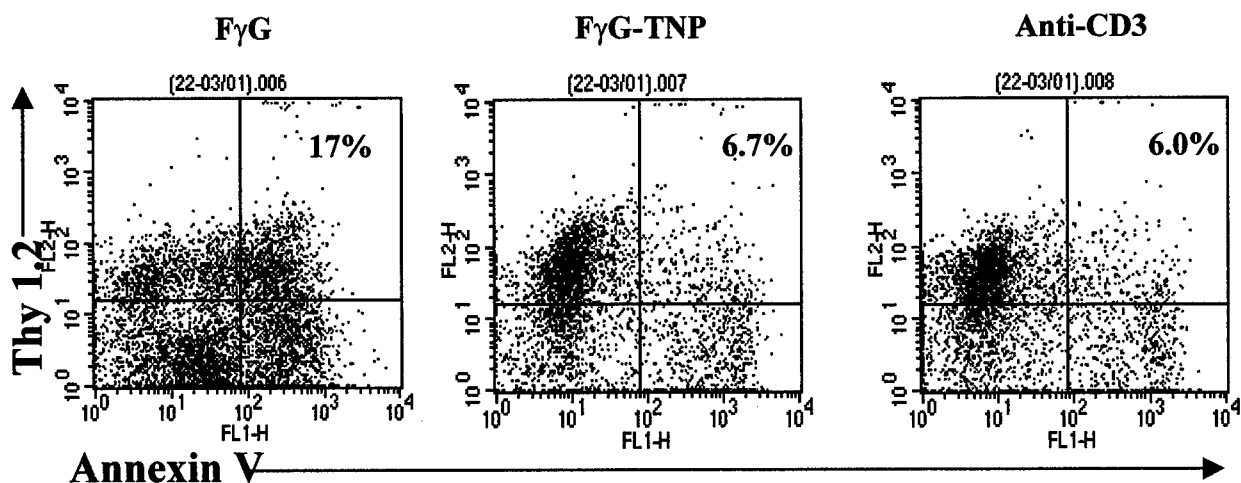
Fig 4. *Rescue from apoptosis (Annexin V staining) in unprimed naïve T cells of Tg8.7 (90%).* Purified T cells were cultured in 24-well plates that had been coated with the indicated reagents. After 72hrs, cells were stained with PE-Thy1.2 and FITC-Annexin V. Necrotic cells were gated out using TOPRO-3 reagent.

48 hr after stimulation   **72 hr after stimulation**

M.W.
32.5–

A   B   C   A   B   C

← Bcl-X$_L$

25 –
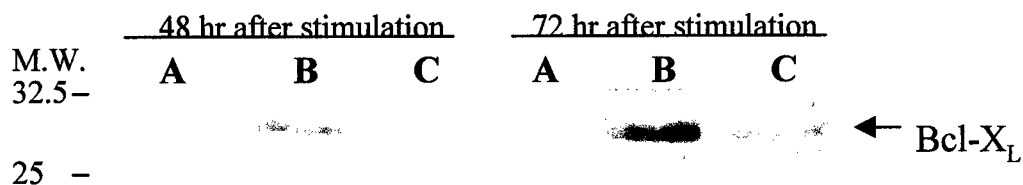
Fig 5. Induction of bcl-X$_L$ protein after stimulation via anti-TNP-CD28-γ CR. Purified T cells were cultured on 24-well plates immobilzed with FγG (10 µg/ml)(**A**), FγG-TNP (10 µg/ml)(**B**) and anti-CD3 (2 µg/ml)(**C**). After 48 and 72 hrs., cytoplasmic lysates were prepared, and the lysates from the same numbers of viable cells (5 x 10^5/lane) in each group were subjected to SDS-PAGE and Western blot analysis.

FγG   **FγG-TNP**   **Anti-CD3**

Thy 1.2

CD25/IL-2Rα
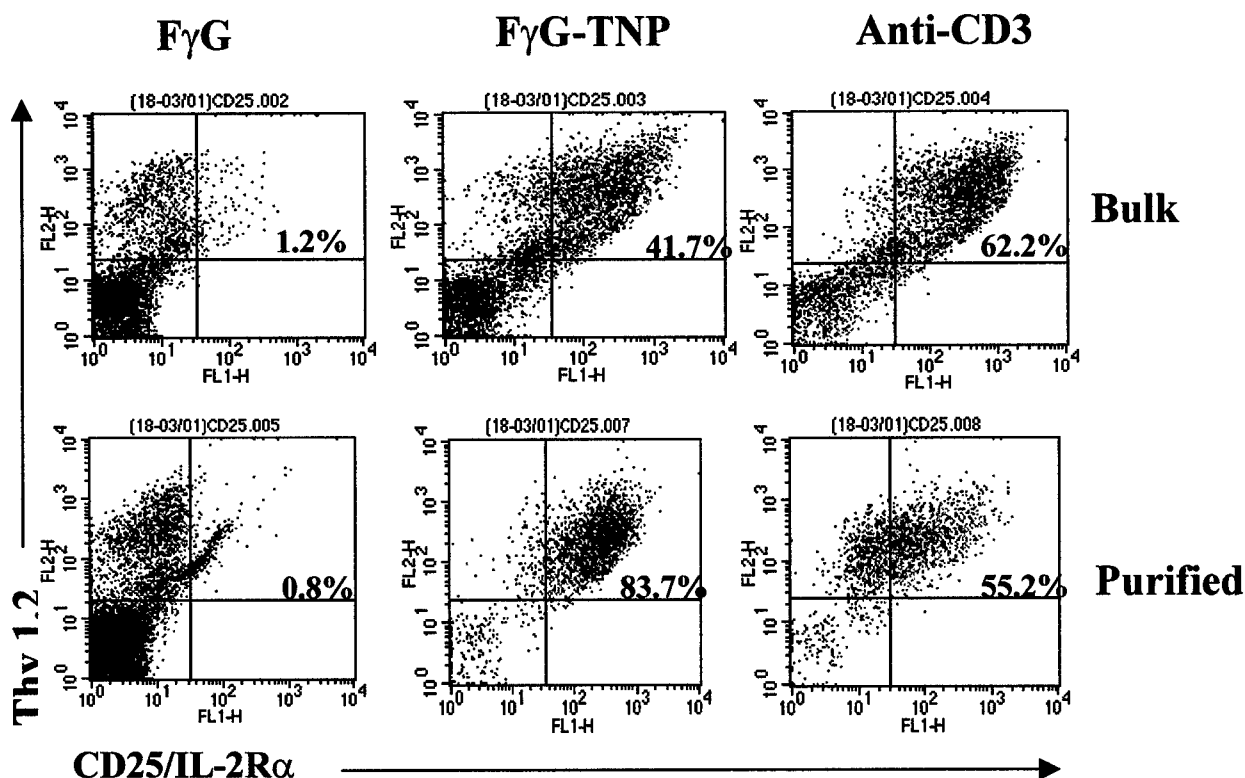
Fig 6. Up-regulation expression of IL-2R α-chain by stimulation via anti-TNP-CD28-γ in unprimed naïve T cells of Tg 8.7-BH mice (60 hr). Similar cultures to those in Fig 4 were performed. Sixty hours later, cells were harvested and stained with biotinylated anti-mouse IL-2R α-chain mAb, followed by FITC-conjugated streptavidin and PE-Thy1.2.
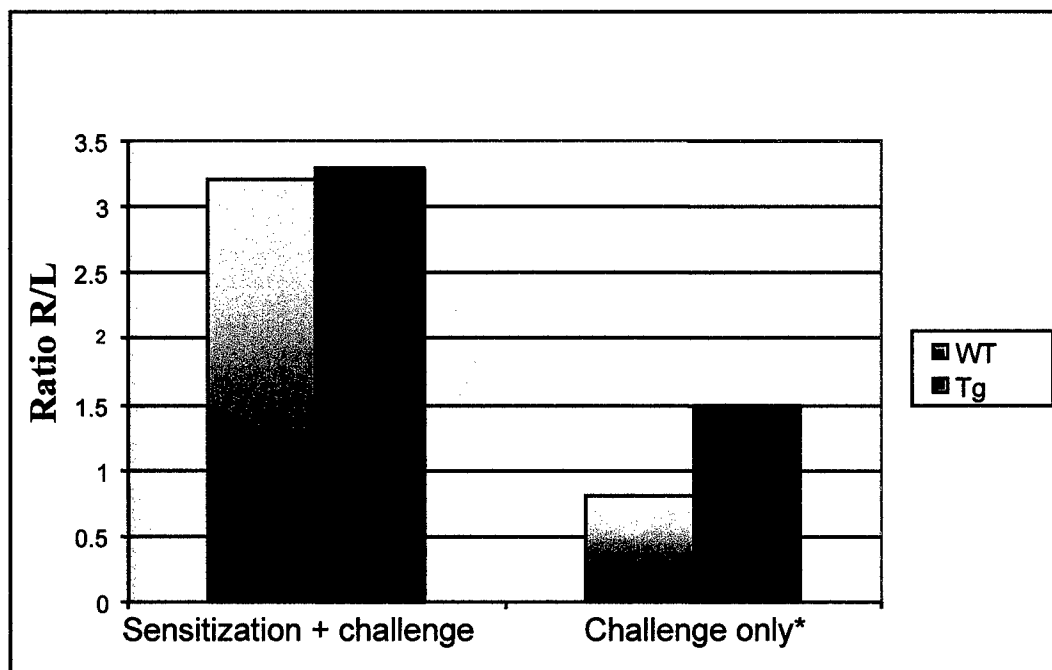
Fig 7. *DTH responses in WT and Sp6-CD28-γ transgenic mice.* All mice were cyclophosphamide pre-treated and then divided in two groups: mice from "sensitization+challenge" group were Ag-sensitized 5 days before challenge. Mice from "challenge only" group were directly challenge with the Ag in the right pinna. Four mice were tested per group. * p<0.005.
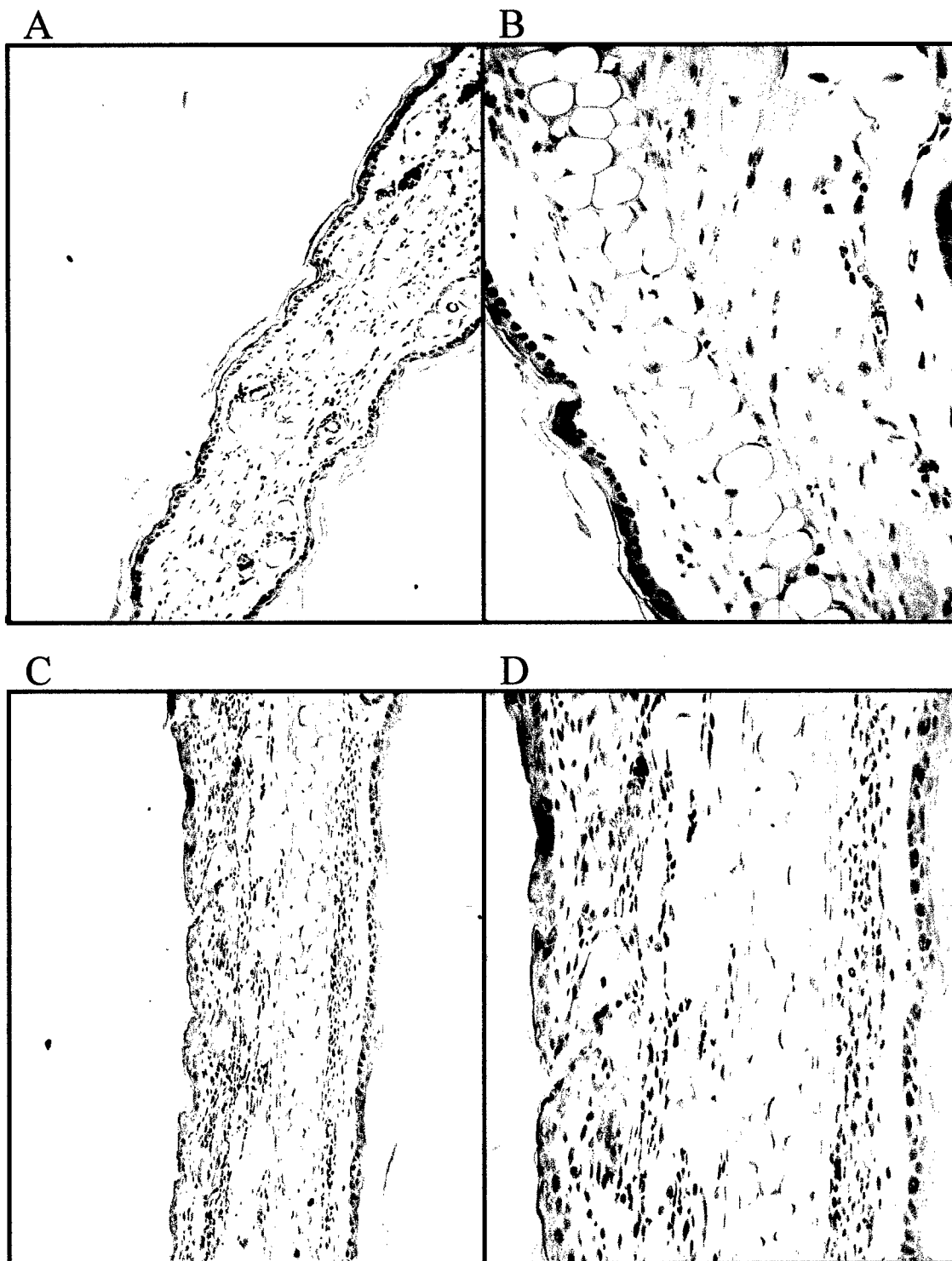
Fig 8.Histology sections of pinnae of Tg 8.7 mouse (representative of "challenge only" group, see Fig 7).Pinnae were fixed in buffered formalin, cut and stained with HE.
Note subepidermal and epidermal infiltration of immflamatory cells, mainly neutrophils and lymphoid and plasma cells in the right pinna. **A** and **B** left pinna, **C** and **D** right pinna.
A and C: x20. B and D: x40.
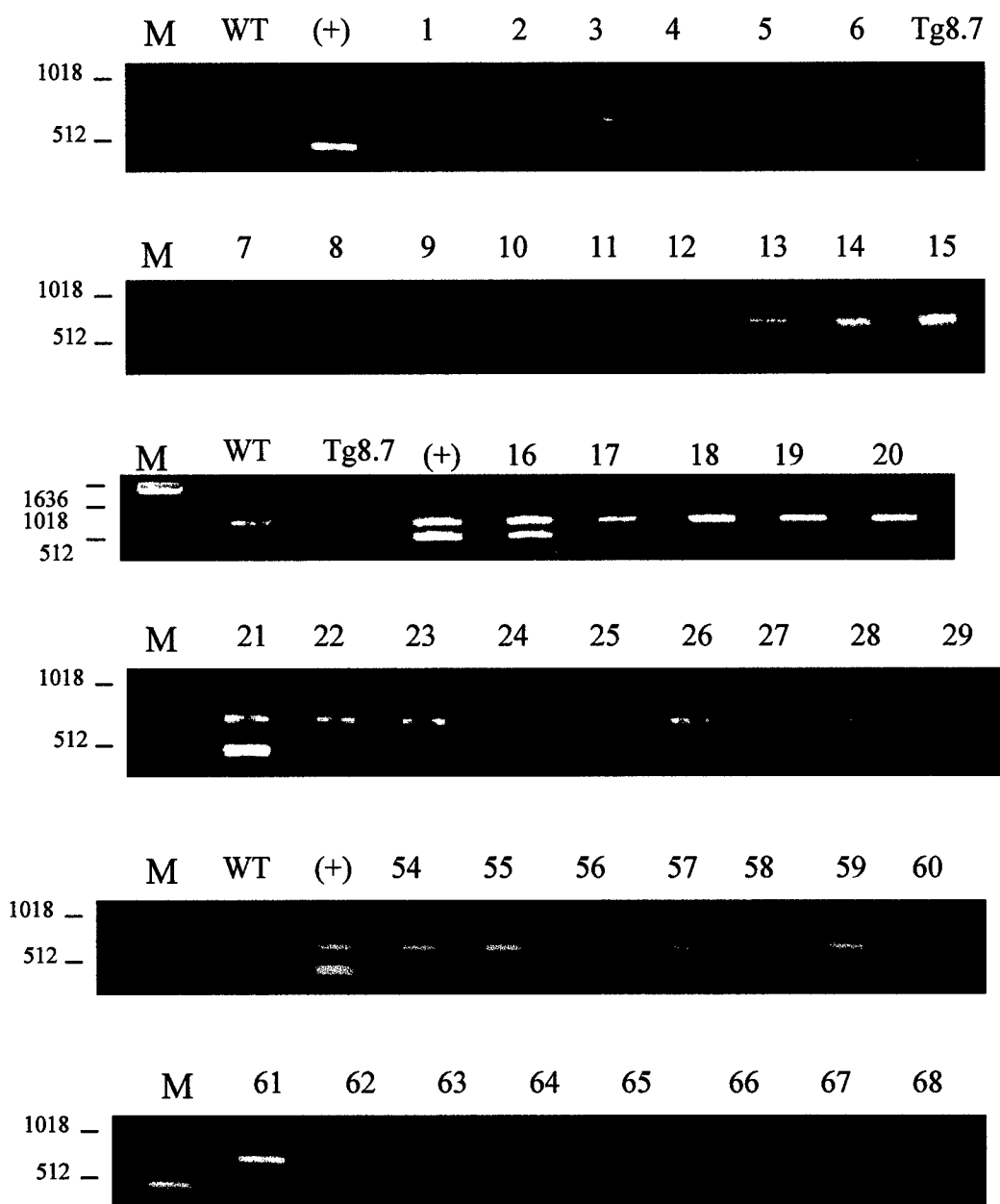
Fig.9. *PCR analysis of transgene integration in TgN29 founder mice.* Representative gels of founders TgN29 1-29 and 54-68, negative controls: CB6F1 genomic DNA (WT) and Tg8.7, positive control (+): CB6F1 genomic DNA doped with closed circular VA-N29-CD28-γ. A 530 bp TgN29 specific sequence was PCR amplified from 0.5 μg of genomic DNA using an upstream primer specific of N29 and a downstream primer located within the γ sequence. PCR products were visualized by agarose gel electrophoresis and EtBr staining.
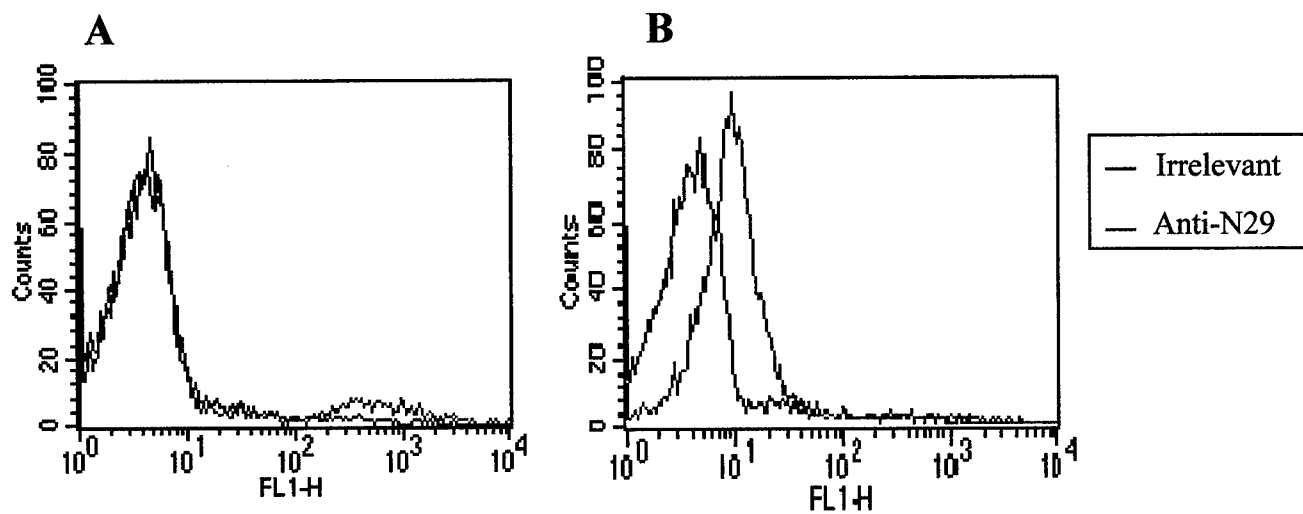
Fig.10. *Surface expression of N29-CD28-γ in T cells of transgenic founder mouse.*
Peripheral blood lymphocytes of WT (A) and Tg (B) mice were stained with Thy1.2, anti-N29
idiotype and irrelevant antibody and subsequently analyzed by two-color flow cytometry.
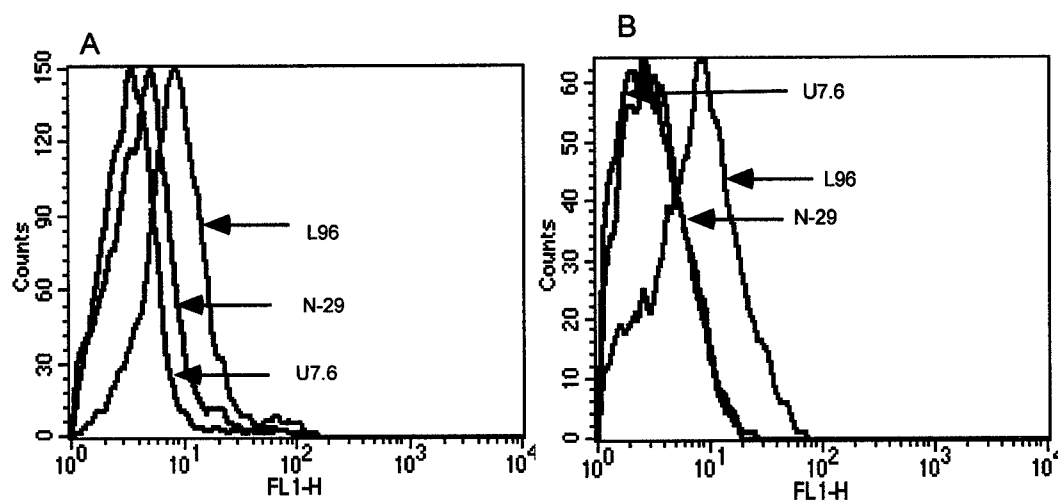Analyzed cells are Thy1.2 gated .

Fig 11. *Expression of the N29 epitopes of HER-2 on CWR22 tumor cells*
A- Tumor from a control animal that was treated with intra-tumoral administration of
lymphocytes bearing an irrelevant receptor (anti TNP) with systemic IL-2.
B- Re-growth of a tumor from an animal that was treated with intra-tumoral administration
of lymphocytes bearing an anti-HER2/N29 receptor with systemicIL-2. Note 20.5%
expression of N29 epitopes on the control tumors (B) in contrast to negligble expression of
this epitopes on tumors that initally responded to the specific treatmentbut susequently regew.
In contrast only a minor reduction in the expression of HER-2 (as indicates by staining with
L96 directed against different epitopes) is noted. Staining with the isotype matched U 7.6
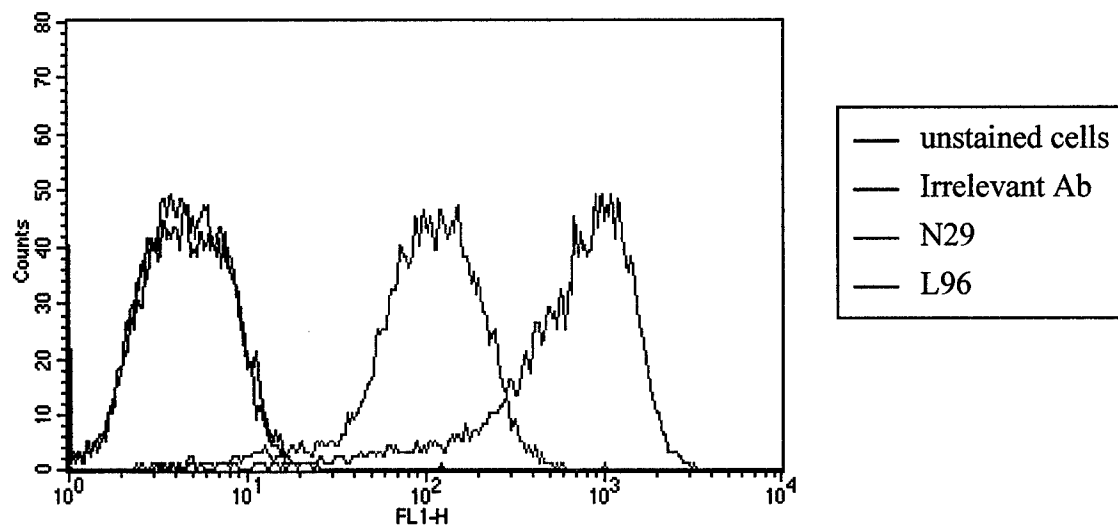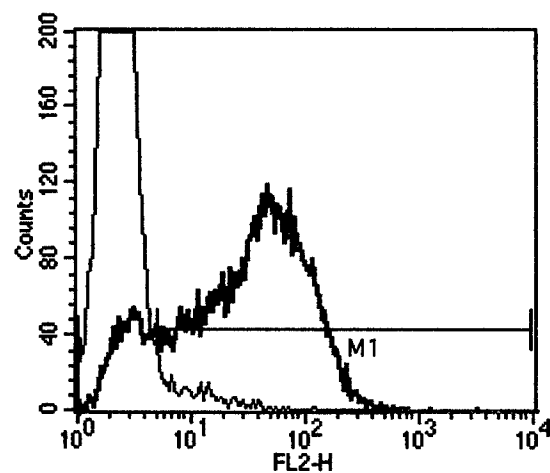antibody directed against irrelevant antigens serves as a background.

Fig 12. *Surface expression of erbB2 antigen on SKBr3 human breast adenocarcinoma cell line.* Flow cytometry analysis of SKBr3 cells stained with N29, L96 and irrelevant antibody.

Key	Name	Parameter	Gate
——	19.03.01.026	FL2-H	No Gate
——	19.03.01.027	FL2-H	No Gate

B16 - 5.5%

B16-N29 -85%

Fig 13. *B16 melanoma expressing human erbB2.*
The B16 melanoma was transfected with Her2/Neu gene and stained with the N29 anti-erbB2 monoclonal antibody and FITC-labeled anti-mouse antibody.
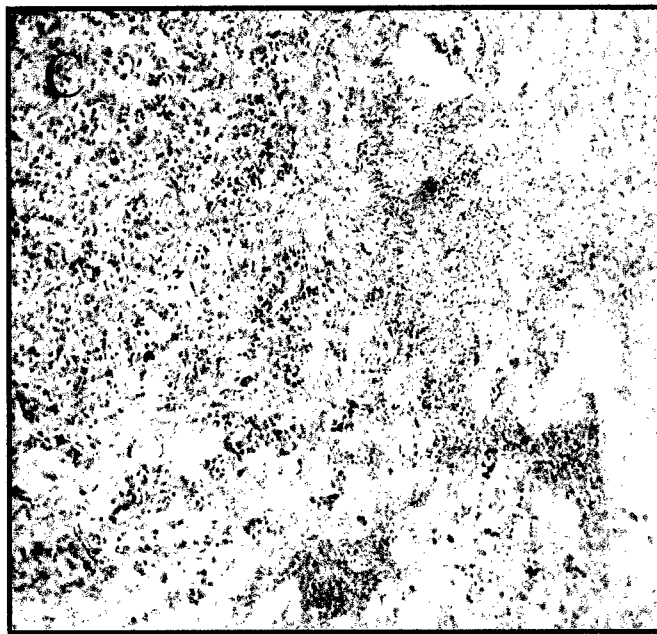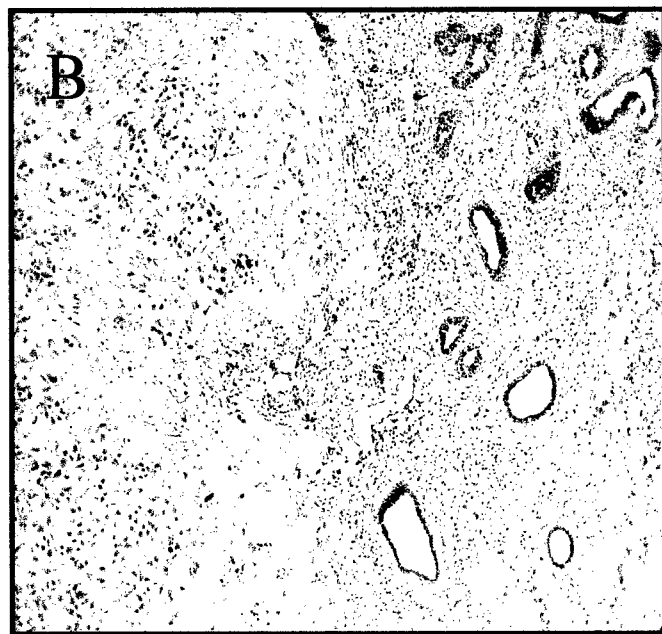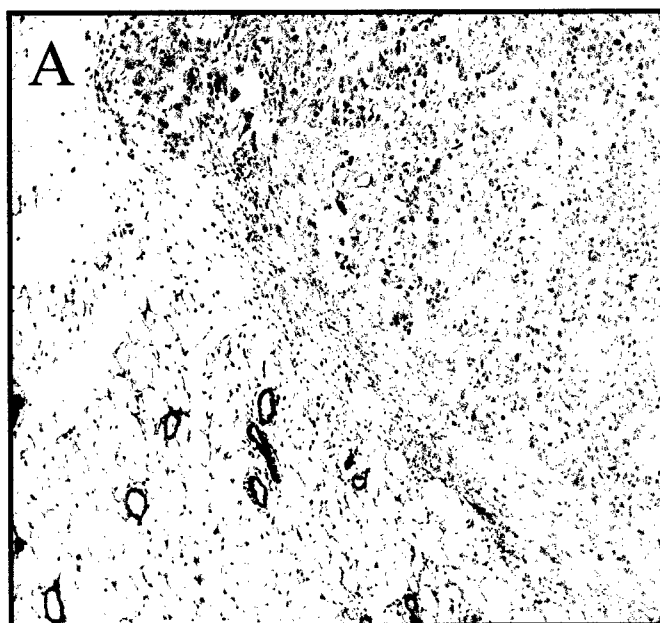
Fig 14. *Histological analysis of SKBr3 xenograft tumors in the mammary fat pad of SCID mice.*
A Non-treated tumor. *B* Intratumoral injection of human erbB2 specific T bodies + IL-2.
C same as B without IL-2.
Note areas of immflamation in xenograft tissue in *B* and *C*.

Review article

Functional expression of chimeric receptor genes in human T cells

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Abstract

Tumor immunotherapy has been limited to date by the poor antigenicity of most tumors, the immunocompromised state of many cancer patients, and the slow tumor penetration and short half-life of exogenously-introduced anti-tumor antibodies. Our group has developed a model immunotherapy system using a chimeric construct containing an antibody V region fused to a T cell activation molecule (T body) introduced by transfection into cytotoxic T cell lines, or populations of activated primary T or natural killer (NK) cells. In this study we have optimized the conditions needed for efficient transduction of human peripheral lymphocytes (PBL) using retroviral vectors pseudotyped with the gibbon ape leukemia virus (GaLV) envelope. Selection of packaging cells producing high virus titers was performed following transfection with constructs containing the green fluorescent protein (GFP), and FACS sorting. As a model chimeric receptor gene we used a tripartite construct consisting of a single-chain anti-TNP antibody variable region linked to part of the extracellular domain and the membrane spanning regions of the CD28 coreceptor molecule and joined at its 5′ end to a gene fragment encoding the intracellular moiety of the γ activation molecule common to the Fcε and Fcγ receptors. Enriched preparations of retrovectors containing this chimeric receptor and the GFP gene could stably and efficiently transduce human PBL co-activated by anti-CD3 and anti-CD28 antibodies. In routine experiments, the transgene was expressed in 35–70% of the human T cells. Such lymphocytes express the chimeric receptors on their surface and upon stimulation with hapten immobilized on plastic they can produce IL-2. Transfectomas activated in this manner also undergo specific proliferation in the absence of exogenous IL-2. Moreover, the transduced lymphocytes could effectively lyse target cells expressing the TNP hapten on their surface. These studies establish the conditions for the optimal transfection of effector lymphocytes to redirect them against a variety of tumor targets. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Chimeric receptor; Retrovectors; T cells; Gene expression; Gene delivery

1. Introduction

Abbreviations: GFP, green fluorescent protein; scFv, single chain Fv; MuLV, Moloney murine leukemia virus; GaLV, gibbon ape leukemia virus; TNP, trinitrophenol; PBL, peripheral blood lymphocytes; NK, natural killer cells

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To overcome the inability of antibodies to eliminate solid tumors on the one hand as well as the lack of specific of anti-tumor T cells in many malignancies our group has pioneered the T body approach (Gross et al., 1989; Eshhar et al., 1993). In the T-body methodology, we have joined the two approaches of adoptive T cell immunotherapy and

antibody therapy to genetically engineer an improved 'immunocytolysin', an antibody V region attached to a cytotoxic T cell signaling molecule and responsible for recognition and activation. Genes for chimeric T cell receptors have been constructed containing the coding sequence of an antibody-derived single-chain Fv directed against a tumor associated antigen, attached to the transmembrane and intracytoplasmic sequences of T cell signaling molecules. These genes are then transfected into cytotoxic T cells, thereby conferring upon them the ability to specifically recognize and kill tumor cells. The scFvR design we have developed combines antibody recognition and T cell signaling in one continuous protein and has been used to endow murine and human effector cells with non-MHC-restricted, antibody-derived specificity. This T-body approach combines the advantage of antibody specificity with the homing, tissue penetration, and target cell destruction mediated by T lymphocytes.

Using this technology, in the past 10 years we have been able to redirect lymphocytes to predefined targets and to endow them with antibody-like specificity. We have designed and developed several receptor configurations, identified the optimal receptor molecules to give a strong effector response, and have chosen a single chain antibody configuration that yields effective antigen binding for virtually all antibodies (Eshhar et al., 1995; Fitzer-Attas et al., 1998). We have transfected effector cells including the mouse MD45 cytotoxic cell line, human NK (Bach and Eshhar, 1995) and rat basophilic cell lines (Bach et al., 1994) and human tumor infiltrating lymphocyte populations.

The use of redirected effector cells has potential in the immunotherapy of virtually any type of cancer in which tumor antigens, not present on normal tissues, are expressed and shared between patients. Using in vivo model systems, lymphocytes transfected with a chimeric receptor of anti-folate binding protein specificity developed in our lab, were shown to eliminate experimental tumors in mice (Hwu et al., 1993, 1995). In addition, we have made a receptor construct containing anti-HER2 antibodies, which is able to mediate cytolysis when transfected into cytolytic mouse hybridoma cells.

For clinical application, patient-derived lymphocytes will be transfected with a chimeric receptor gene encoding an scFv specific to an antigen expressed by the tumor. After expansion in vitro, such genetically engineered cells will be reinfused into the patient where they are expected to undergo activation at the tumor site and reject the tumor, either by direct cytotoxcicity and/or by causing a local inflammatory response. One of the major technical limitations to this scenario is inefficient gene transfer into T cells. For stable gene expression, the only vectors approved for use in humans are adeno-associated and certain retroviral based vectors. While adeno-associated viruses (AAV) fail to infect T cells, some promising results have been obtained using Moloney murine leukemia virus (MuLV)-based vectors for gene delivery into T cells (Mavilio et al., 1994; Bunnell et al., 1995). It was demonstrated that transduction of human T cells can be augmented by pseudotyping the MuLV with the envelope protein (env) of the Gibbon ape leukemia virus (GaLV) (Bunnell et al., 1995; Lam et al., 1966). For integration into the recipient cell genome, retroviral-mediated gene transfer requires dividing cells. A significant enhancement of gene delivery into human T cells has been recently obtained by combining T cell activation using anti-CD3 plus anti-CD28, and transduction in the presence of fibronectin derived peptide (Pollok et al., 1998, 1999; Dardalhon et al., 2000).

Here we increased the efficacy of functional expression of chimeric receptor genes in primary human T lymphocytes by a procedure that combines optimal T cell activation with improved retrovectors. Such a procedure can be readily applied to the immunotherapy of human cancer using ex vivo transduction of patient lymphocytes with chimeric receptors derived from antibodies specific to surface antigens expressed by the tumor in question.

2. Materials and methods

2.1. Retroviral packaging cell lines

The packaging cell lines used include the ecotropic GP+E-86, the amphotropic PA317 (obtained from ATCC), and PG13, which expresses the GaLV env (Miller et al., 1991). 293T cells were a generous gift from R.A. Willemsen (Daniel den Hoed Cancer Center, Rotterdam. The Netherlands). All cells were

cultured in DMEM medium (GIBCO-BRL) supplemented with 10% FCS (GIBO-BRL, Paisley, UK), L-glutamine solution (2 mM), sodium pyruvate 1 mM, 100 Units/ml penicillin and 100 μg/ml streptomycin (Biological Industries, Israel).

2.2. Antibodies and reagents

Anti-human CD3 antibody was purified from the OKT3 hybridoma cell (ATCC) culture supernatant. Anti-CD28.2 was obtained from PharMingen, (San Diego, CA). SP6, an anti-TNP monoclonal antibody (mAb), and 20.5, an anti-Sp6 idiotype mAb, were both provided by G. Kohler (Max-Planck Institute for Immunology) (Rusconi and Kohler, 1985). Fluorescein-labeled anti-mouse Ig antibodies were purchased from Jackson Immuno Research Laboratories Inc (West Grove, PA).

2.3. Transfection/infection

2.3.1. 'Ping-pong' method

First, 3×10^5 GP+E-86 cells and 2×10^5 PA317 cells were co-cultured in a 100-mm plate (NUNC, Roskilde, Denmark). A day later, medium was changed and transfection was performed by adding 20 μg of plasmid DNA in $CaPO_4$ (Mammalian Transfection Kit, Stratagene, La Jolla, CA). After 24 h, the plate was washed with PBS and supplemented with fresh medium. When the culture reached near confluence (24–48 h post transfection), viral supernatant was collected for infection. For infection, 2×10^5 PG13 or 293T cells were plated in a 100-mm plate; the next day, the medium was replaced with 5 ml of viral supernatants in the presence of 4 μg/ml of Polybrene (Sigma) at 37°C, 7.5% CO_2 for 7 h. After 48 h, the infection efficiency was evaluated by monitoring GFP expression (see below). All vector-containing retroviral supernatants described in this study were harvested after a 24-h incubation of near-confluent packaging cells grown in 5 ml fresh medium in a humidified incubator at 32°C, 7.5% CO_2

2.4. Activation of lymphocytes

Peripheral blood lymphocytes (PBL) from healthy donors were isolated by centrifugation through Ficoll Paque Plus (Pharmacia Biotech, Uppsala, Sweden), and cultured in RPMI 1640 medium (GIBO-BRL) supplemented with 10% FCS, L-glutamine solution 2 mM, 100 Units/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol.

Cells (10^6/ml in a well from a 24-well plate) were stimulated for 2 days on Falcon non-tissue culture-treated 24-well plates pre-coated with anti-CD3 plus anti-CD28 antibodies. Immobilization of the antibodies was performed by adding 0.5 ml/well of antibody (1 μg/ml PBS of each of the antibodies/ well) to the wells overnight at 4°C; the plates were washed with PBS and blocked with 1% BSA in PBS for 20 min at 37°C.

2.5. Lymphocyte transduction

Activated lymphocytes were harvested from the stimulating plate, washed and plated on a RetroNectin™ (FN) (Takara Shuzo Ltd. Otsu, Japan) coated plate at 0.5×10^6/well with 2.8 ml of viral supernatant supplemented with 50 U/ml of IL-2 (recombinant human IL-2 Chiron, Amsterdam, The Netherlands). After 4–6 h at 37°C, 7.5% CO_2, the viral supernatant was gently removed and replaced with RPMI–FCS+50U/mlIL-2 and cells were incubated overnight at 37°C, 5% CO_2. The same transduction process was repeated on the next day. On the day following the second infection, lymphocytes were harvested by vigorous flushing and washing of the wells. The cells were re-suspended in RPMI–FCS medium with 150 U/ml of IL-2, and incubated in 37°C, 5% CO_2. FN-coated plates were prepared by incubating non-tissue culture-treated 24-well plates (Falcon) with 1 ml/well of FN (12 μg/ml) overnight at 4°C, washed with PBS and blocked with 1% BSA in PBS for 20 min at 37°C.

2.6. Flow cytometry

Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Green fluorescent protein (GFP) was measured on FL1 (488 nm excitation and 530 nm emission filter). Viability was determined by assessing propidium iodide (PI) (Sigma) staining using FL2 at 488 nm excitation and 585 nm emission. Expression of scFv (Sp6) on the surface of the cells was evaluated by

immunofluorescent staining using anti-Sp6 mAb (20.6) and rhodamine–phycoerythrin (PE) labeled anti-mouse Fab′ antibody, measured on FL2. Cells were sorted on a FACSsort Plus (Becton Dickinson) according to GFP fluorescence. Cloning of packaging cells from single cell/well positive cells sorted directly into 96-well plates.

2.7. Cell proliferation and cytotoxicity assays

The 96-well plates (non tissue culture treated, NUNC) were coated overnight with 2 μg/well of antigen (TNP-FγG) at 4°C, washed with PBS and blocked with 1% BSA on the following day. Alternatively, for stimulation, various targets were irradiated (12 000 R) and TNP-modified as described previously (Gross et al., 1989). Transduced lymphocytes were washed twice and resuspended in culture medium RPMI–FCS with 50 μM 2-mercaptoethanol. Cells were left for 2–4 h in the incubator to eliminate any remaining IL-2. Transduced lymphocytes (1×10^5/well) were plated on antigen (TNP-FγG), coated plates or cocultured with TNP-modified target cells. At different time points, 50 μl of the cell culture was removed to be tested for cell proliferation by the MTT assay (Mosmann, 1983).

Evaluation of the cytotoxicity of transduced lymphocytes was performed by the ^{51}Cr release assay as described before (Gross et al., 1989).

3. Results and discussion

3.1. Vector and chimeric gene constructs

In order to establish the optimal conditions for transduction of primary human T cells, we tried several systems reported to be efficient for gene delivery of chimeric receptor genes. The BULLET system described by Weijtens et al. (1998) was attempted first; we then evaluated the STITCH vector, a modification of the BULLET system which was developed by the same group (Willemsen et al., 2000). This system involves the transient transfection of three plasmids to produce a transducing retrovirus. One plasmid codes for the envelope protein, which could be from an ecotropic virus, an amphotropic virus, or from the gibbon ape leukemia virus (GaLV). Another plasmid supplies the gag and pol proteins. The transfer vector with the LTRs and packaging signal carries an insert with the gene of interest (Fig. 1). In our experiments, this insert was specially designed. The scFv used in our initial experiments was from an antibody with specificity to trinitrophenol (TNP). This facilitates the testing of the genetically modified lymphocytes for their ability to kill a wide variety of cells as any cell can be made a target simply by treatment with picryl chloride to label the cell surface with the TNP group. Another advantage of this scFv is that we have available an anti-idiotypic monoclonal antibody for detecting by FACS expression of this scFv on the cell surface. In the construct used, the co-stimulatory molecule CD28 is genetically fused to this scFv. This provides spacing of the scFv from the cell surface and permits the formation of heterodimers with endogenous CD28 molecules. The CD28 molecule has previously been used in chimeric receptor constructs (Vallina and Hawkins, 1996; Finney et al., 1998). In addition, CD28 signaling has been shown to prevent apoptosis of the lymphocytes. Since CD28 is homologous to CTLA4 and since the structure of CTLA4 has been determined we have employed the latter as a model for the structure of CD28. Alignment of their amino
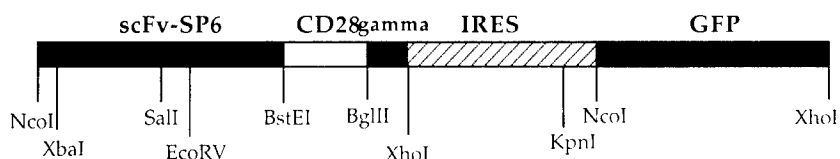

Fig. 1. Structure of the chimeric receptor-GFP gene used in this study. Sp6-scFv is a single chain Fv containing the sequence of the leader, V_L, linker and V_H of the anti-TNP Sp6 antibody, CD28 sequence contains the CD28 hinge region, transmembrane region, and cytoplasmic region. The γ chain sequence contains the cytoplasmic region (starting from the QVR at a BglII site). This deletes CRLKI at the beginning of the cytoplasmic region of the Fcε receptor γ and contains the signaling portion of the receptor. IRES is an internal ribosomal entry site (IRES) that permits expression of the GFP from the same transcript as the chimeric receptor.

acid sequences to that of a mouse heavy chain reveals that the sequence in CD28 IHV matches the *BstE* II site sequence in our single chain VTV (Holm and Sander, 1996). Therefore, the junction was made so that the junction is at a valine which is conserved between the end of the framework 4 beta sheet of the variable region of the antibody (Kabat numbering 111) and a valine which is just after the last beta sheet in CTLA4.

Human CD28 was cloned from PBLs and Jurkat cells using the following primers:

#7966 *BstE* II primer for homodimer CD28
5′ CCGGTCACCGTGAAAGGGAAACACCTTT-
GTCC
#7967 reverse 3′ primer
5′ CGCTCGAGGTGTCAAGATCTATAGGCTG-
CGAAGTCGCGTGG

In this way the single chain is attached to the CD28 hinge region, transmembrane region, and cytoplasmic region.

The γ chain cytoplasmic region (starting from the QVR at a *Bgl*II site) was next spliced to the end of the CD28 sequence. This deletes CRLKI at the end of the transmembrane or the beginning of the cytoplasmic region of the Fcε receptor γ chain (Spencer et al., 1993) (Fig. 1). This contains the signaling portion of the receptor and completes the gene for the chimeric T cell receptor.

To enable better tracking of retroviral expression, the gene for green fluorescent protein (GFP) was placed downstream of the chimeric receptor gene separated by an internal ribosomal entry site (IRES) to permit expression of the GFP from the same transcript as the chimeric receptor. Retroviruses with a single transcript containing a desired gene and GFP driven by an IRES have been made previously (Aran et al., 1998; Levenson et al., 1998). In our case, the GFP gene was placed in the retroviral vector pSAM-EN (Morgan et al., 1992) in place of the drug resistance gene, and the chimeric receptor gene was inserted into the cloning site before the IRES. Lack of a second promoter avoids the problem of promoter interference. It also enables linked expression so that GFP fluorescence may be used to assess expression of the scFv. Transduced cells can be observed visually by fluorescence microscopy, thereby permit-

ting the tracking of transduced cells in vivo (Persons et al., 1997; Bagley et al., 1998).

3.2. Establishment of stable vector producing cell lines

Retrovirus-containing supernatants prepared from the 293T cells as described above gave up to 15% transduction of fresh 293T cells as estimated by the proportion of cells exhibiting GFP fluorescence (data not shown). The percentage and level of gene expression in this transient system varied and depended on the transfection efficiency and culture conditions. To obtain a more reproducible system, we have attempted to establish a stable vector-producing cell line by selection for high titer retrovector producing clones. Fig. 2 schematically describes the protocol we have employed for these studies. Briefly, the chimeric receptor gene incorporated in a BULLET or STITCH vector was transfected into the amphotropic PA317 and ecotropic GP+E-86 lines, which were co-cultured in order to increase the viral titer. The supernatant obtained was used to infect a series of packaging cell lines whose supernatants, containing the pseudotyped retrovector, were titered on 293T cells and in parallel tested for their ability to infect activated human PBL (as described in Materials and methods). In several experiments,
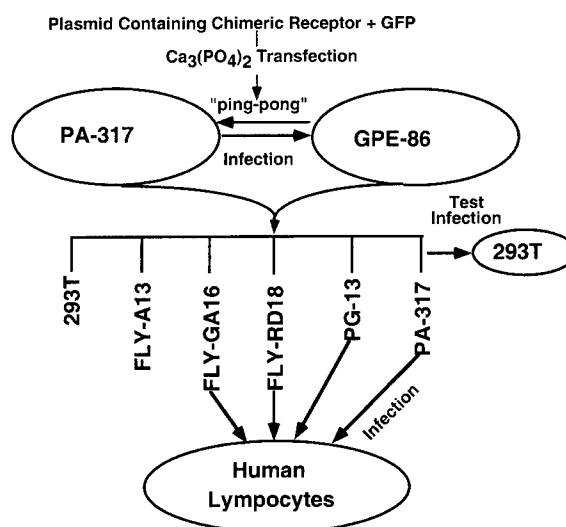

Fig. 2. A diagram of the protocol used for the generation and selection of a high titer packaging cell lines.

the PG13 containing the GaLV envelope, was highly infected, produced the highest titer of virus and resulted in the best gene transfer into the human T cells (data not shown). It was previously shown that the receptor to the GaLV envelope, GLVR-1 is increased following T cell stimulation (Lam et al., 1966). The heterogeneous population of packaging cells obtained was sorted for GFP expression by FACS and the sorted population was further sub-cloned (Fig. 3). As can be seen in the figure, virtually all the cells expressed GFP following a single cycle of sorting.

3.3. Infection of primary human T cells

For optimal transduction of human peripheral blood lymphocytes with retroviruses, the lymphocytes must be activated. In our protocol, they are grown in RPMI with fetal calf serum without IL-2 with plastic bound anti-CD3 and anti-CD28 antibodies for 48 h. They are then infected with supernatant containing virus from the packaging cell line on plates coated with recombinant fibronectin fragments (Retronectin™). The infection is performed in the presence of IL-2 for 5–7 h, twice over 48 h as described in Materials and methods. Low concentrations of IL-2 were used to prevent the propagation of natural killer cells. Higher concentrations are used for the production of lymphokine-activated killer (LAK) cells (Jadus et al., 1988). RetroNectin™ is a recombinant fibronectin fragment which contains the connecting segment, cell binding domain and heparin binding domain (Hanenberg et al., 1996). Its significantly enhances the infection efficacy, since both the retroviral particles and T cells bind to it (T cells through the VLA integrin, the expression of which is increased following anti-CD3/CD28 stimulation).

Several days following transfection, the cells were phenotyped for surface markers and for expression of the chimeric gene using anti-idiotypic antibody and evaluated for expression of GFP. The percentage of transfected cells, 3–4 days following transduction was in the range of 35–70% and remained stable for about 10–14 days. After this period, the proportion of GFP positive cells began to slowly drop unless they were re-stimulated by anti-CD3/CD28, PHA or allogeneic cells. Phenotypically, the transfected cell population consisted of about 30% CD4, 70% CD8

and 15% CD56 positive cells. The population of NK cells (CD56+) dropped during culture in the low IL-2 concentration.

Since throughout all the selection and sorting procedures we followed GFP expression, and since the chimeric receptor gene and GFP were in two separate cistrons, it was important to check whether the expression of GFP truly indicated chimeric receptor expression. Fig. 4 shows the FACS pattern of transduced T cells that were stained with anti-idiotypic antibody against the Sp6 anti-TNP scFv and also analyzed for GFP expression.. It is clear that most of the GFP expressing cells also expressed the chimeric receptor on their surface.

3.4. Functional expression of the chimeric receptor genes in human T cells

In vivo, effector lymphocytes redirected with chimeric receptors are expected to reach their destination, undergo specific stimulation at the target site to either kill the target or secrete cytokines that in turn will cause inflammation and result in elimination of the tumor. Ideally, the effector lymphocytes may differentiate into memory cells which will stay on-guard and prevent reappearance of the tumor in question. To check the ability of the chimeric-receptor expressing human T cells to perform these functions in vitro, we tested their ability to undergo proliferation and to produce cytokines in response to specific antigen as well as their ability to kill specific target cells.

Fig. 5 depicts the results of an experiment in which T cells expressing the SP6-scFv-CD28-gamma chimeric receptor (anti-TNP specific) were triggered for specific proliferation following stimulation on antigen (TNP-FγG) coated culture-wells and the removal of exogenously supplied IL-2. While lymphocytes did not proliferate following 3 days of culture in the absence of IL-2, in the presence of TNP-FγG (hapten/protein ratio >2) they propagated quite well for at least 1 week. In the presence of antigen excess (TNP$_{58}$-FγG) cells started to die following 7 days, most likely because of overstimulation or antigen-induced cell death (AICD) that was not prevented by the presence of CD28 in the signaling receptor. Notably, the cell proliferation, and IL-2 production (data not shown), was triggered
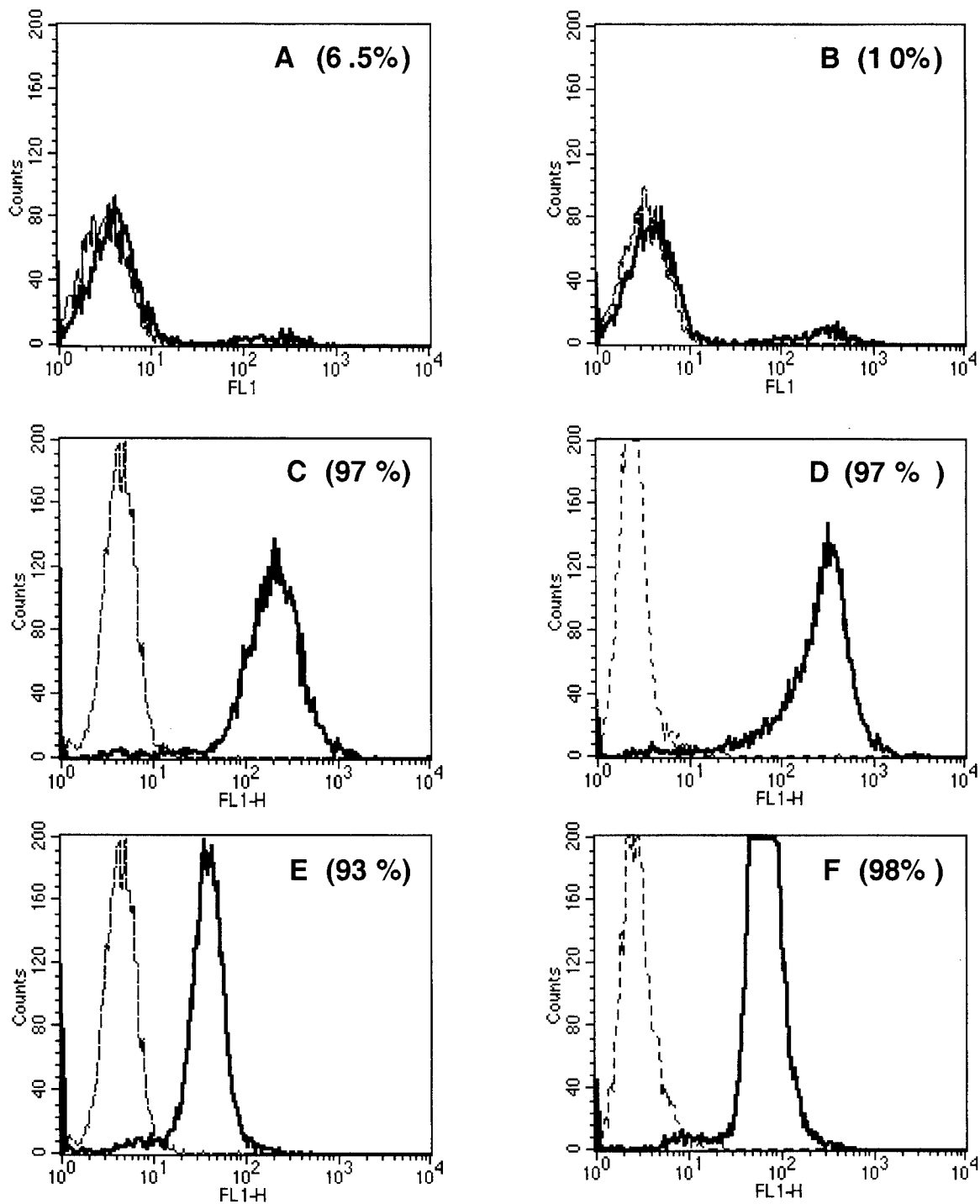
Fig. 3. GFP fluorescent pattern of two packaging cells at different stages of their production. A, C, E represent cell lines derived from the amphotropic PA317 cell; B, D, F represent cell lines derived from the PG13 cell containing the GaLV envelope. A, B-packaging cells 24 h after their transduction by cell-free supernatants obtained from the 'Ping-Pong' step (Fig. 2). C, D represent the GFP expression in bulk-sorted packaging cells. E, F represent clones selected for high vector titer from the bulk-sorted population.
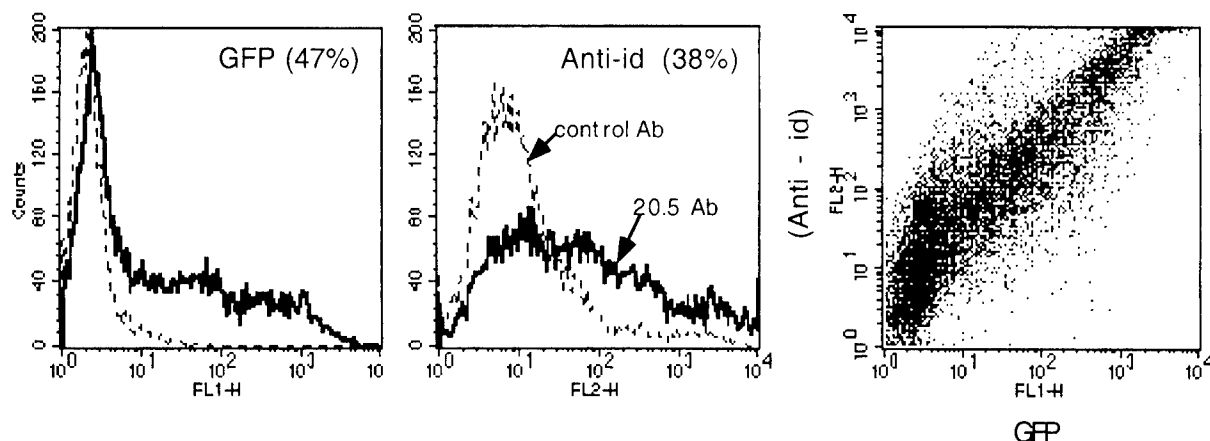
Fig. 4. Co-expression of the chimeric receptors and GFP transduced human T cells. Fluorescent pattern of GFP expression (FL1) and surface expression of the chimeric receptor stained with biotin–anti-idiotypic antibody (20.5) and avidin–phycoerythrin.

by the chimeric receptor in the absence of any other stimulatory and co-stimulatory signal, showing that both signal I and II can be induced by our tripartite receptor. The T cells bearing the chimeric receptors could also specifically and efficiently kill various TNP-modified target cells regardless of their species or tissue origin (Fig. 6). The genetically engineered lymphocytes were able to efficiently cytolyse their targets following 3 weeks culture in the presence of IL-2, even in the absence of preactivation before the

cytotoxicity assay. At this stage, the level of non-specific LAK-mediated killing was low.

Taken together, the results described above demonstrate that under the conditions used, PBL-derived T cells can be efficiently transduced to express high levels of chimeric receptors. The chimeric receptor could induce the T cells to specifically proliferate and produce cytokines as a result of specific stimulation by antigen alone without additional stimulation. The genetically engineered cells maintain their abili-
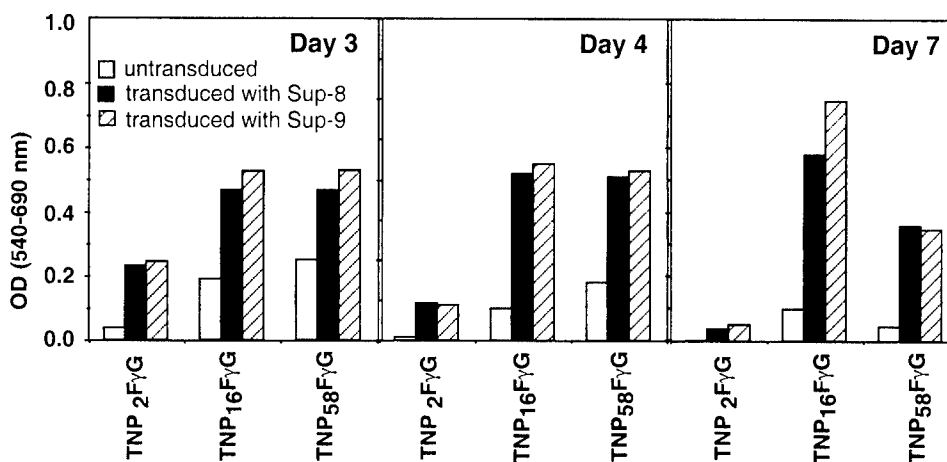

Fig. 5. Proliferation of chimeric receptor expressing lymphocytes stimulated with antigen. T lymphocytes transduced with retrovector preparations made of two packaging clones 8 and 9, as well as non-transduced cells were cultured in the absence of IL-2 on plastic-immobilized antigens containing different hapten groups (TNP) per FγG carrier. Cell proliferation at different days in culture was monitored by the MTT colorimetric assay. Note that the culture medium was changed at day 4.
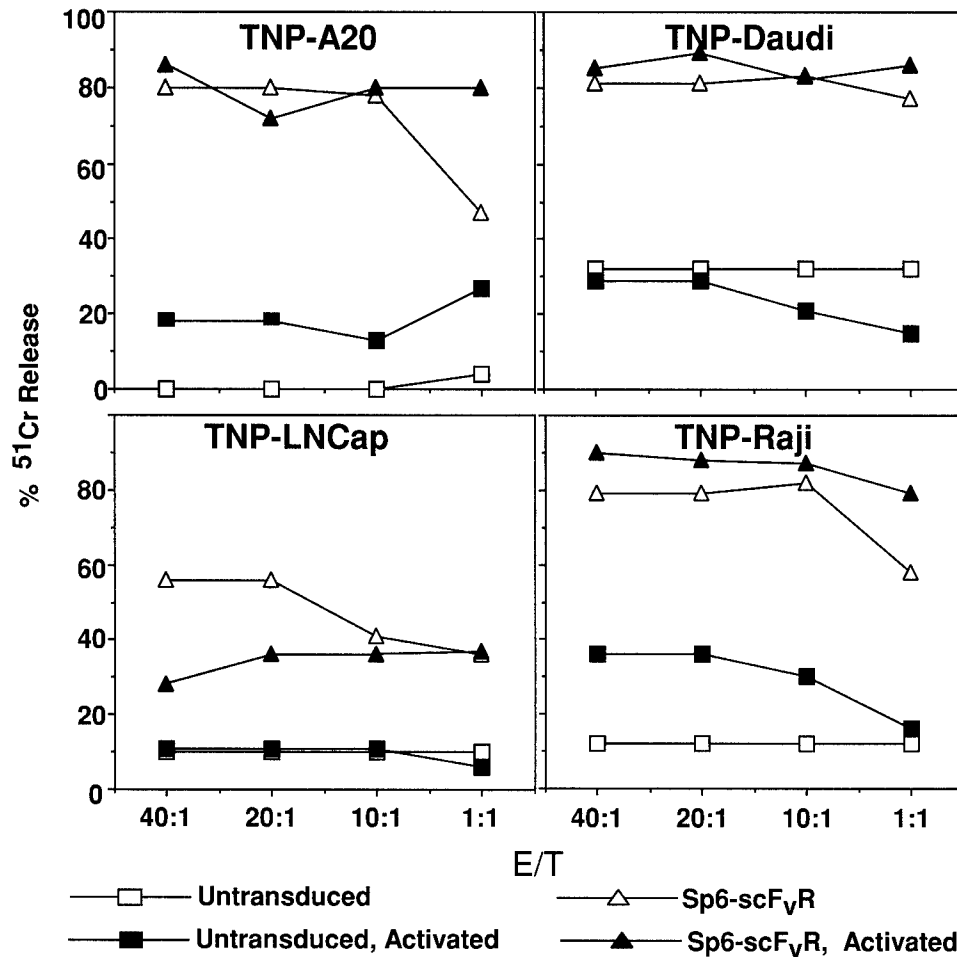
Fig. 6. Specific target cell cytotoxicity mediated by the chimeric receptor expressing lymphocytes. Lymphocytes were harvested 3 weeks after their transduction with chimeric receptor containing vectors. Part of the cells were reactivated on anti-CD3 and anti-CD28 antibodies 24 h before the assay. As target cells served various cell lines modified and unmodified by TNP. Specific ^{51}Cr release from the targets following incubation at different ratios with effector cells (E/T ratio) was determined following 7 h.

ty to kill specific target cells for at least 3 weeks in tissue culture, a period long enough to produce sufficient cells for patient treatment. These results set the grounds for in vivo testing of the T-body approach in animal models for cancer immunotherapy and later in patients.

Acknowledgements

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